A novel in bioassay for (xeno-)estrogens using transgenic zebrafish

Legler, J.; Broekhof, J.L.M.; Brouwer, A.; Lanser, P.H.; Murk, A.J.; van der Saag, P.T.; Vethaak, A.D.; Wester, P.; Zivkovic, D.; van der Burg, B.

published in
Environmental Science and Technology
2000

DOI (link to publisher)
10.1021/es0000605

document version
Publisher's PDF, also known as Version of record

Environ. Sci. Technol. **2000**, *34,* 4439−4444

A Novel in Vivo Bioassay for (Xeno-)estrogens Using Transgenic Zebrafish

JULIETTE LEGLER,[†,‡]
JOSÉ L. M. BROEKHOF,[‡]
ABRAHAM BROUWER,[†]
PETER H. LANSER,[‡]
ALBERTINKA J. MURK,[†]
PAUL T. VAN DER SAAG,[‡]
A. DICK VETHAAK,[§] PIET WESTER,[#]
DANICA ZIVKOVIC,[‡] AND
BART VAN DER BURG*[,‡]

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands, Division of Toxicology, Wageningen University and Research Centre, P.O. Box 8000, 6700 EA Wageningen, The Netherlands, Ministry of Transport, Public Works and Water Management, National Institute for Coastal and Marine Management, P.O. Box 8039, 4330 EA Middelburg, The Netherlands, and National Institute of Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands

Adverse trends in the reproductive health of male fish, including testis abnormalities and intersex gonads, have been increasingly reported over recent years. These effects have been associated with the exposure of fish to natural, synthetic, and xenobiotic estrogens present in the aquatic environment. A novel in vivo test system using transgenic zebrafish has been developed to rapidly determine the effects of estrogenic chemicals on critical life stages and sensitive target organs in the fish. In the transgenic zebrafish, an estrogen binding sequence linked to a TATA box and luciferase reporter gene was stably introduced. Binding of a substance to endogenous estrogen receptors (ER) and the subsequent transactivation of the ER result in luciferase gene induction that is easily measured in tissue lysates. Exposure to estradiol (E2) during juvenile stages of the transgenic zebrafish revealed the period of gonad differentiation to be the most responsive early life stage. In adult male transgenic zebrafish, the testis was the most sensitive and responsive target tissue to estrogens. Partial sequences of zebrafish estrogen receptor subtypes α and β were cloned for the first time and were found to be differentially expressed in developing fish and tissues of adult male zebrafish. The transgenic zebrafish assay is a promising new tool to rapidly determine the estrogenic potency of chemicals *in vivo.*

Introduction

Endocrine disruption is an issue that has raised public concern and is on the political and research agenda of governments worldwide. Reports of chemicals in the environment that can mimic the actions of endogenous estrogens, thereby disturbing normal endocrine functions and causing male reproductive dysfunction in humans and wildlife (reviewed in refs *1*−*3*), are increasing. In wild fish populations, intersex (the simultaneous presence of both male and female gonadal characteristics) and testis abnormalities have been found in a high proportion of male fish sampled in rivers, estuaries, and coastal waters (*4*−*6*). These feminizing effects have been associated with exposure to environmental levels of natural, synthetic, and xenobiotic chemicals (xeno-estrogens) in the aquatic environment. Natural estrogens include the female hormones estradiol, estrone, and estriol. Synthetic estrogens are pharmaceutical chemicals designed to mimic the action of natural estrogens, such as the birth control pill component ethinylestradiol as well as diethylstilbestrol. Xeno-estrogens can be defined as environmental and industrial pollutants that are not designed to be used as estrogens but nevertheless can evoke effects via the estrogen receptor signal transduction pathway. Laboratory exposure of male fish to (xeno-)estrogens has resulted in the synthesis of high levels of the estrogen-inducible yolk precursor protein vitellogenin (VTG) as well as inhibited testicular growth, testis abnormalities, and formation of intersex gonads (reviewed in ref *7*).

Regulations aimed at determining a substance's potential to disrupt endocrine systems have proven to be extremely difficult because estrogenic substances often have very different chemical structures, hampering their analysis and risk assessment on a structural basis. Tests to determine estrogenic effects on laboratory animals are available but are laborious, time-consuming, costly, and may require large amounts of animals. As an alternative, simpler screening methods such as in vitro reporter gene assays have been developed, allowing large-scale screening of chemicals (reviewed in ref *8*). These assays make use of the fact that the receptor for estrogens is a transcription factor that induces transcription of target genes after binding to specific DNA sequences in their promoter. However, major drawbacks of such cell lines are, compared to in vivo measurements in animals, that important aspects of in vivo functioning such as metabolic conversion and breakdown can be missed. Moreover, no assessment can be made of the vulnerability of critical life stages, such as developing embryos, to the hormonal disrupting compounds.

With this in mind, we have developed a novel test system for (xeno-)estrogens using zebrafish in which an estrogen responsive reporter gene has been stably introduced. Using transgenic reporter zebrafish, the direct effects of estrogenic chemicals on estrogen-sensitive tissues can be readily determined during various stages of sexual development. Because of the large number and rapid development of offspring, transgenic zebrafish can combine the advantages of in vitro and in vivo systems to provide a rapid and simple in vivo model to screen for hormonally active substances. In addition, zebrafish genetics and early development have been widely studied (*9*), and it is a recommended freshwater fish species for toxicity testing (*10*). Using these transgenic fish, we show reporter gene induction in vivo by (xeno-)estrogens following short term exposure, demonstrating the presence of highly responsive estrogen receptors in sexually differentiating juvenile fish. Of the wide range of organs tested in adult male fish, the reproductive organs appear to be the main target tissue for estrogens.

* Corresponding author phone: 31 30 2121923; fax: 31 30 2516464; e-mail: bvdb@niob.knaw.nl.
[†] Wageningen University and Research Centre.
[‡] Netherlands Institute for Developmental Biology.
[§] National Institute for Coastal and Marine Management.
[#] National Institute of Public Health and the Environment.

10.1021/es0000605 CCC: $19.00
Published on Web 09/08/2000

Experimental Section

Generation of Transgenic Zebrafish. Zebrafish were maintained at 27 °C on a 14 h light/10 h dark photoperiod and were fed brine shrimp *Artemia salinas* four times daily. To generate transgenic zebrafish, zebrafish embryos were microinjected with 15 pg of the supercoiled DNA construct pEREtata-Luc (*11*) prior to first cleavage essentially according to Stuart et al. (*12*). In total, about 1600 embryos were injected. At 24 h post fertilization (hpf), 940 (56%) of embryos survived and were individually tested for luciferase expression by immersion in 200 μL of a nontoxic luciferin substrate solution (20 mM tricine, 1 mM $(MgCO)_4MgOH_2$-$5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP, pH 7.8)) in 96-well plates. Embryos were assayed for luciferase activity in a scintillation counter (Top Count, Packard). Embryos with luciferase expression (about 500 embryos or 55% of the survivors) were selected and reared to sexual maturity (approximately 3 months). Potential founder transgenic fish were crossed with wild-type zebrafish. F_1 offspring at 24−48 hpf were pooled in eppendorf tubes (about 60 embryos per tube) and lysed overnight at 55 °C in 500 μL lysis buffer containing 100 mM Tris·HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 100 μg/mL proteinase K. Genomic DNA was extracted by addition of 500 μL isoproponal, mixing and centrifugation at 14000 rpm for 10 min. DNA was dissolved in 200 μL of TE buffer (10 mM Tris-Cl, 1 mM EDTA (pH 7.5)). PCR was performed in a Biometra PCR on 1 μL DNA samples using primers within the luciferase gene (upstream primer GGTCCTATGATTATGTCCGG and downstream primer GGCCTTTATGAGGATCTCTC). The following conditions were used for 32 PCR cycles: denaturation at 95 °C for 5 min, annealing at 56°, and extension at 72 °C for 1 min. Offspring of a total of 142 adult fish were analyzed, of which 42 were identified as transgenic founders (= 30% germ-line transmission). Confirmation of stable integration was carried out by performing Southern blots on genomic DNA. Of the 42 transgenic founders, two independent lines were identified with similar inducible luciferase activity following 48-h exposure of 4−5 week old offspring to 1000 nM E2. All studies presented here were carried out with one line with the highest expression of luciferase. This line, deemed 1.31, showed 13% germ-line transmission of the luciferase gene from F_0 to F_1 generation. In the F_2 and F_3 generations obtained by crossing transgenics with wild-type zebrafish, 50% of offspring were transgenic, demonstrating Mendelian inheritance of the luciferase gene.

Exposure Studies. Exposure studies with transgenic zebrafish were carried out with heterozygous F2 en F3 adult (3−6 months of age, weight range 500−1000 mg) and F3 juvenile (<6 weeks of age) fish of the highly expressing transgenic 1.31 line. Fish were exposed to ligands or solvent controls in dimethyl sulfoxide (DMSO) or ethanol not exceeding 0.01% via the water phase in glass Petri dishes or aquaria for 48 or 96 h with daily renewal. Following sacrifice, tissues were dissected from adult fish. Juvenile fish less than 28 days post fertilization (dpf) in age were pooled. Juvenile fish older than 21 dpf were sampled individually. Fish (tissues) were lysed in 500 μL Triton-lysis buffer (pH 7.8) containing 1% Triton X-100, 25 mM glycylglycin, 15 mM MgSO4, 4 mM EGTA, and 1 mM DTT and frozen at −80 °C. Samples were thawed on ice, homogenized with an Eppendorf micropestle, and centrifuged (15 min, 14 000 rpm at 4 °C). Luciferase activity was assayed on duplicate samples of 25 μL using a luminometer (LUMAC) with automatic injection of 100 μL luciferine substrate solution containing 33 mM DTT according to manufacturer's specifications. Protein correction was carried out according to the method of Bradford (*13*). For fish older than 21 dpf, 3−4 transgenic fish were exposed per group. Experiments were repeated at least twice.

ER Isolation. To isolate zebrafish ER (zfER) subtypes, total RNA was isolated from nonexposed female gonads using acid guanidium thiocyantate-phenol-chloroform extraction. Poly-(A)+ RNA was isolated by oligo(dT) microbeads (Miltenyi Biotec). Random primed double-stranded cDNA was synthesized using a cDNA synthesis kit (Gibco B.R.L). To isolate zfER-α, degenerate primers were chosen with homology to other fish ER-α sequences: upstream C-domain DNA binding region primer HYGVW (CAT/CTAT/CGGA/T/G/CGTA/G/C/TTGG) and downstream E-domain ligand binding region primer I/MKCKNK (TTA/GTTT/CTTA/GCAT/CTTCAT). A second set of degenerate primers was designed for nested PCR (upstream primer VGMMKG (GTA/T/G/CGGA/G/T/CATGATGAAA/GGG) and downstream primer MSNKGM (CATA/G/T/CCCT/CTTA/GCTCAT)) resulting in an 840 nucleotide sequence with highest homology (84%) with channel catfish ER-α (*14*). To isolate zfER-β, degenerate primers described elsewhere to isolate a putative ER-β in the Japanese eel (*15*) encompassing the C and E domain were used (upstream primer: GACTAC/TATGTGC/TCCC/TGCGAC and downstream primer: GTGAC/GCGTCCAGCATCTCCAG). A second set of nested degenerate primers (KCYEVGM (AAATGTTATGAAGTT/C/A/GGGA/C/T/GATG) and KGMEHLS (ACTAAGATGTTCGATA/G/C/TCCT/CTT)) were used, resulting in an 857 nucleotide fragment showing highest homology (80%) with the Japanese eel putative ER-β isoform (*15*). The nucleotide sequences of the zfERα and -β partial fragments can be accessed in the EMBL Nucleotide Sequence Database (Accession numbers AJ275910 and AJ275911, respectively).

ER Distribution using RT-PCR. Using the partial zfER-α and β sequences described above, specific primers were designed to detect zfER-α (forward primer: GGGCGTTCTGTCAGGCGTAAG and reverse primer CAGGCGATCATGTGGACGAGT) and zfER-β (forward primer: CGTAACCCCCAAATCAGAGACAGC and reverse primer: ATCCTCAGGAGTCTGTGGCAAACT) in cDNA prepared from RNA isolated from developing stages of nonexposed juvenile zebrafish and tissues from adult males. Specific primers from zebrafish glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer: GGCTCCTTTGGCAAAGGTCA and reverse primer: TGGCAGGTTTCTCAAGACGG) were used as an internal control for RT-PCR reactions (*16*). Prior to synthesis of random primed double-stranded cDNA, DNA was removed from RNA samples by treating 10 μg RNA with 1 Unit RQ1 RNAse-Free DNAse (Promega) in a volume of 100 μL of Reaction Buffer (Promega). After incubation of samples for 30 min at 37 °C, the reaction was stopped with 25 μL of Stop Solution (Promega) and 125 μL of a phenol:chloroform: isoamyl alcohol (25:24:1) solution. RNA was precipitated by adding 825 μL of 100% ethanol, mixing, and centrifuging for 30 min at 14 000 rpm. The RNA pellet was then washed twice with 70% ethanol and dissolved in 20 μL of water. cDNA was synthesized using 1 μg of RNA per reaction using the Superscript cDNA synthesis kit (Gibco B. R.L). RNA samples were first incubated for 3 min at 65 °C, quickly chilled on ice, and briefly centrifuged. The reverse transcriptase (RT) reaction was carried out in a volume of 20 μL containing 1 μg RNA, 100 ng oligo(dT) primer, 5 mM dNTP mix, 0.1 mM DTT, RT reaction buffer, and 200 units Superscript II reverse transcriptase (Gibco B.R.L.) for 90 min at 37 °C. Following incubation, 30 μL water was added to the samples for a total volume of 50 μL cDNA. PCR reactions were carried out using 5 μL cDNA samples in a total volume of 50 μL, containing 2.5 mM $MgCl_2$, PCR reaction buffer (1X, Eurogentec), 0.4 mM each of dATP, dCTP, dGTP, and dTTP (Gibco B.R.L.), and 1.5 units of TAQ polymerase (Eurogentec). Primers for zfERα, zfER-β, and GAPDH were used a concentration of 20 pmol per reaction. PCR reactions were carried out in a Biometra PCR using 35 cycles of amplification for each

reaction. The following conditions were used: denaturation at 95 °C for 5 min, annealing at 53 °C (zfERα), 68 °C (zfERβ), or 58 °C (GAPDH), and extension at 72 °C for 1 min. As a positive control, a sample of zebrafish liver cDNA giving the same amount of product as 10 000 copies of specific plasmid DNA was included in the amplification reaction. No PCR products were found in RNA or water controls.

Immunohistochemistry. Adult male transgenic and wild-type fish were fixed in 4% paraformaldehyde containing 10% EDTA and embedded in paraffin. Immunohistochemistry was performed on 5 μm sections following antigen retrieval by heating sections four times for 5 min in 10 mM citrate buffer, pH 6. Following blocking with 20% swine serum, sections were incubated with a 1:200 dilution of an affinity purified rabbit polyclonal anti-luciferase (Promega), followed by avidin-conjugated swine anti-rabbit Ig, and avidin-peroxidase with a biotin bridge. Reactivity was visualized using diaminobenzidine + H_2O_2 (Sigma).

Results and Discussion

We developed transgenic zebrafish stably expressing pERE-tata-Luc, an estrogen responsive luciferase reporter gene regulated by three estrogen response elements (ERE) upstream from a TATA box. The pEREtata-Luc construct and activation by estradiol in vitro have been described elsewhere (*11*). Since enhancer regions other than the ERE are absent in this construct, it very specifically responds to estrogen receptor (ER) activation. Compounds activating endogenous estrogen receptors in target cells lead to receptor binding to the EREs and consequently activate transcription of the luciferase gene. Following short term exposure of transgenic zebrafish to (xeno-)estrogens, the production of luciferase protein can be easily assayed by preparing tissue lysates and measuring light activity following addition of the enzyme substrate luciferin.

Estrogen Receptor Gene Activity Is Developmentally Regulated in Transgenic Zebrafish. Expression of luciferase in transgenic zebrafish was determined in developing life stages following 48 h exposure in water to 1000 nM E2 (Figure 1a). Range finding toxicity tests carried out prior to these experiments under the same exposure periods established that this concentration did not cause acute toxicity (data not shown). In embryos less than 28 days post fertilization (dpf), luciferase activity could be easily assayed in lysates of pooled embryos. In fry between 28 and 35 dpf of approximately 1 cm length, luciferase activity was assayed in lysates of individual embryos. Luciferase induction increased with age and stage of gonad differentiation, ranging from 4-fold in 1 day old embryos (1 dpf) to 100 to 300-fold induction in 35 dpf juvenile transgenic fry relative to vehicle-exposed controls (Figure 1a). Gonad differentiation in the zebrafish commences at about 2 weeks of age in zebrafish (*17*). Clear evidence of female ovarian and male testicular gonad differentiation has been observed in fry at about 28 dpf (Wester, unpublished results). Our results indicate that transgene induction correlates with the sexual development of the zebrafish.

As transgene expression is regulated by the ER, we determined the timing of expression and distribution of the zebrafish ER (zfER). We isolated specific fragments of ER subtypes α and β in zebrafish ovarian cDNA and designed specific primers for semiquantitative PCR (see Experimental Section). It should be noted that the PCR reactions with primers for zfER-β were more efficient than with the zfER-α primers (Figure 1b, control), indicating that the absolute levels of zfER-α mRNA may be underestimated. zfER-α mRNA was detected at all stages tested in the developing embryo and fry, though the expression is highest late in sexual differentiating stages (35 dpf) (Figure 1b). zfER-β mRNA was also detected at all stages, though was low at 14 dpf, the stage of the onset of gonad differentiation. From 28 to 35 dpf, ERβ mRNA expression increased, coinciding with high luciferase induction (Figure 1a). Our results suggest that estrogen receptors are very active during sexual development. Therefore, the period of gonad differentiation may be very sensitive to disruption by estrogenic compounds.

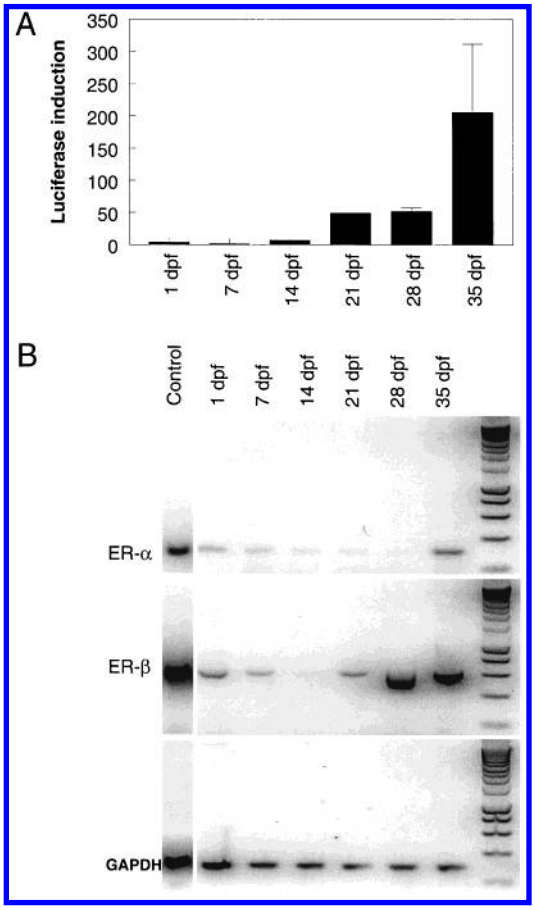

FIGURE 1. (A) Luciferase activity in developing life stages of transgenic zebrafish exposed for 48 h to 1000 nM 17β-estradiol (fold induction in light units/μg protein relative to vehicle exposed controls) and (B) stage-related expression of zebrafish estrogen receptor type ER-α and ER-β mRNA in nonexposed developing life stages (dpf = days post fertilization). "Control" lane shows PCR product from liver cDNA (10000 copies) amplified under the same conditions. In Figure 1(A), age given indicates age at initiation of exposure. Bars show the luciferase induction in pools of embryos from 1 to 21 dpf: 1 dpf: *n* = 20; 7 dpf: *n* = 10, 14 dpf: *n* = 10; 21 dpf: *n* = 5. For embryos of 28 and 35 dpf, bars show mean luciferase induction in individual fry (*n* = 4); error bars show standard error of the mean.

Ligand Sensitivity of Reporter Gene Induction in Juvenile Fish. To determine the sensitivity of juvenile fish to E2, dose−response studies were carried out with transgenic 35 dpf fry (Figure 2a). A nominal concentration of 0.1 nM E2 resulted in 7 ± 4-fold induction following 96 h exposure (Figure 2a), indicating that the juvenile fish undergoing sexual differentiation may be sensitive to E2. The concentration of 0.1 nM E2 is a realistic concentration that may be encountered in aquatic systems in the environment (*18*). Some natural, synthetic, and xenobiotic estrogens were also tested for their potential to induce luciferase in juvenile transgenic zebrafish. Following 48 h exposure to a nominal concentration of 1000 nM, the natural estrogens 17α-estradiol and estrone as well as the synthetic estrogens diethylstilbestrol and ethinylestradiol induced luciferase at levels ranging from 70 to 300 times that of nonexposed controls (Figure 2b). Importantly, the principle DDT isomer o,p′-DDT, a known environmental estrogen in mammals (*19*) and fish (*20*), also induced

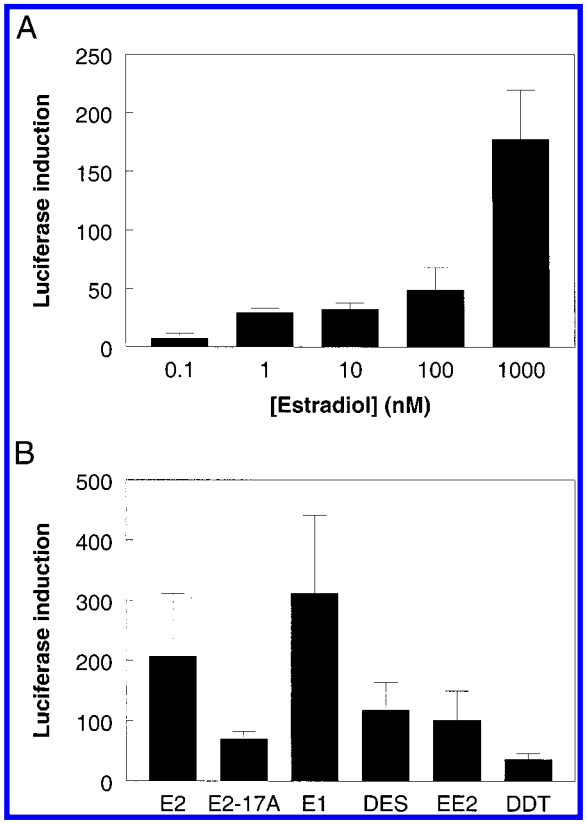

FIGURE 2. Luciferase activity (fold induction in light units/μg protein relative to vehicle exposed controls) in juvenile (35 days post fertilization) transgenic zebrafish exposed for 96 h to (A) increasing doses of 17β-estradiol (E2) and (B) 1000 nM of the estrogenic compounds 17α-estradiol (E2-17A), estrone (E1), diethylstilbestrol (DES), ethinylestradiol (EE2), and o,p′-DDT (DDT). Bars show mean luciferase induction in individual fry ($n = 3-4$); error bars show standard error of the mean.

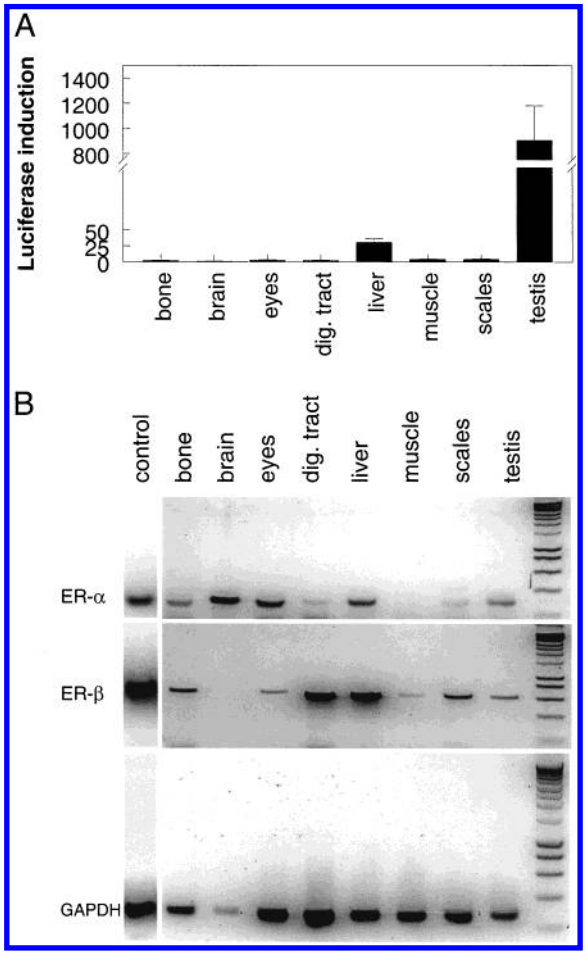

FIGURE 3. (A) Luciferase activity in tissues of adult male transgenic zebrafish exposed for 48 h to 1000 nM E2 (fold induction in light units/μg protein relative to vehicle exposed controls) and (B) tissue-related expression of zebrafish estrogen receptor type ER-α and ER-β mRNA in nonexposed adult male zebrafish. "Control" lane shows PCR product from liver cDNA (10 000 copies) amplified under the same conditions.

luciferase activity to a level around 40 times that of the solvent control (Figure 2b). It should be noted that relatively high variation was found in the response of individual transgenic fish to estrogens. The outbred genetic background of these heterozygous transgenic fish may contribute to the variation in estrogenic response observed since differences in genetic background may greatly influence the response to estrogens (*20*). In addition, both males and females undergoing gonad differentiation were assayed together at this stage, as their phenotypic sex is not yet apparent. It was found that males and females respond differently to exogenous estrogens (see below).

Estrogen Reporter Gene Induction Is Highly Tissue Specific. To determine the tissue distribution of transgene expression, we studied the response of adult, sexually mature, male transgenic zebrafish to estrogen treatment (Figure 3a). Surprisingly, dramatically high transgene expression of up to 1000-fold was observed in the testis following 48 h exposure to 1000 nM E2 (Figure 3a). In addition, luciferase activity was also prominent in liver (30-fold) and slightly induced in the scales (4-fold) and muscle (3.6-fold). Slight or no significant luciferase induction was found in heart, brain, eye, or bone tissue. In control males exposed to vehicle (DMSO or ethanol) alone, luciferase was not induced, indicating extremely low levels of endogenous estrogens. In control adult transgenic females of 3−6 months of age, however, background levels of luciferase in liver and ovaries were high, reaching approximately the same level as liver in E2 exposed males. High background levels of luciferase in transgenic females are likely due to high levels of circulating endogenous estrogens. Exposure to E2 in adult females did not result in luciferase induction above this elevated background level (data not shown).

Analysis of the zfER mRNA expression pattern in various tissues of the adult male zebrafish confirmed the presence of both zfER-α and ER-β (Figure 3b) in tissues showing high luciferase induction such as testis and liver (Figure 3a). Tissues that did not show high luciferase induction such as brain, bone, digestive tract, and eyes, however, expressed varying amounts of both receptors (Figure 3b). Differences in the expression of the internal control GAPDH do not reflect differences in the amount of tissue amplified per reaction. GAPDH is differentially expressed in various organs, with lower expression in the brain. We therefore did not correct for GAPDH variation throughout the samples. The differences in ER mRNA expression and activation of luciferase between various tissues may be explained by differences in levels of receptor protein or coactivators necessary for ER-mediated transcriptional activation. It is also possible that the luciferase response to E2 in some tissues was limited due to the tissue-specific bioavailability of the ligand, perhaps partly due to the short exposure period.

Ligand Specificity and Tissue Distribution of Testicular Reporter Gene Induction. To gain further insight in the possible significance of the testicular estrogen receptors, we characterized the sensitivity of the transgenic testis to estradiol. A dose−response related luciferase induction to

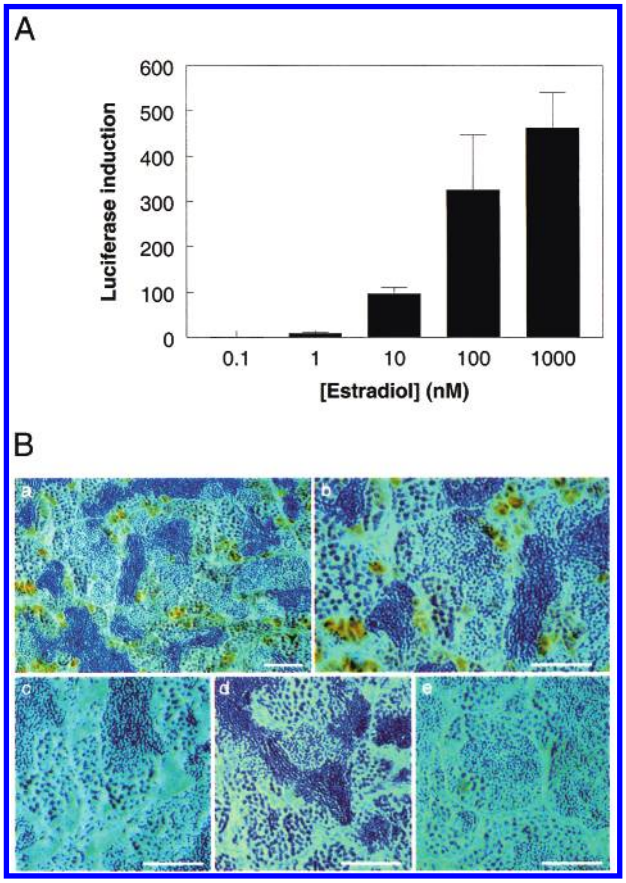

FIGURE 4. (A) Luciferase activity (fold induction in light units/μg protein relative to vehicle exposed controls) in testis of adult transgenic zebrafish exposed for 96 h to increasing doses of E2. (B) Luciferase protein expression in testis of adult transgenic zebrafish exposed for 48 h to 1000 nM E2. Immunohistochemical staining of paraffin-embedded sections using polyclonal anti-luciferase antibody, showing (a, b) expression of luciferase localized in clusters of primary spermatogonia and Sertoli cells. No background staining is found in (c) non-E2-induced transgenic testis; (d) negative controls without primary antibody; and (e) wild-type nontransgenic testis (Bar = 50 μM).

E2 was measured in testis of adults exposed to nominal concentrations of 0.1 to 1000 nM for 96 h (Figure 4a). During this short exposure period, 1 nM E2 already resulted in induced luciferase activity in the adult testis. To localize luciferase-producing cells in the testis, we incubated sectioned tissues of E2-exposed transgenic zebrafish with a polyclonal antibody against luciferase (Figure 4b). Immunohistochemistry of the testis revealed positive staining in the Sertoli cells and early spermatogonia (germ cells) but not in the differentiated spermatocytes and spermatids (Figure 4b-a,b). No staining was found in noninduced transgenic or wild-type controls (Figure 4b-c, 4b-e) as well as transgenic controls without luciferase antibody (Figure 4b-d). Though little is known about the cellular localization of ER subtypes in fish testis, ER has been detected in the testis of goldfish (*22*) and channel catfish (*14*). Localization of luciferase-producing cells in the E2-induced transgenic testis is consistent with the ER distribution found in rodent testis, in which differential expression of ER-α and -β has been found in various cell types, including Sertoli cells and spermatogonia (*23, 24*). At the moment, the functional significance of highly responsive estrogen receptors in male reproductive tissues in unclear. However, luciferase induction in the spermatogonia suggests that estrogens may play a role in the maturation of germ cells. Induction of luciferase in the Sertoli cells may also be consistent with a role in testis size and sperm production.

Our results demonstrate that the testis may be the one of the most sensitive direct target organs for estrogenic pollutants. Although more detailed dose−response studies are necessary, our results with the transgenic zebrafish also suggest that the liver was 2 orders of magnitude less sensitive to exogenous estrogens than the gonads. Recent studies have demonstrated an estrogen receptor in cytosolic and nuclear fractions of the testis of the marine teleost Atlantic croaker (*Micropogonias undulatus*) which binds estrogens and xenoestrogens with higher affinity than the hepatic ER (*25*). The occurrence of intersex gonads in wild populations of fish, which is associated with exposure to (xeno-)estrogens (*4*), also suggests the sensitivity of the gonads to environmental estrogens. We favor the hypothesis that estrogens can directly influence testicular functioning and may even directly regulate the occurrence of intersex. Our future studies aim at testing this hypothesis, thereby testing the validity of the transgenic zebrafish model. Further studies will also be carried out to compare the usefulness of the transgenic zebrafish assay with another widely used biomarker for estrogenic effects in fish, namely the induction of the estrogen-regulated liver protein vitellogenin (*26*).

In conclusion, we have developed a new in vivo model that has the potential to rapidly determine the estrogenic mode of action of chemicals during development and further throughout sexual differentiation in fish. In addition, we can determine whether these chemicals exert their effects directly on the testis or other tissues during adult stages. Thus the magnitude, tissue distribution, and sensitive period of estrogenic effects can be determined in situ. As a complement to the wide variety of in vitro assays currently available to screen compounds for estrogenic activity (*8, 11*), this transgenic model more realistically reflects the availability, kinetics, and metabolism of compounds in vivo. In comparison to long-term reproduction tests, the transgenic zebrafish model may also offer a more rapid and specific means for screening substances with a suspected estrogenic mode of action. The transgenic zebrafish bioassay is a promising new tool in the field of environmental contaminant monitoring and risk assessment of new and existing chemicals.

Acknowledgments

We thank Prof. Dr. J. Koeman for his critical appraisal of the manuscript. The dedicated care of the zebrafish by B. van de Broek, A. Ditewig, E. van Asselt, M. van Soest, and T. den Ouden is much appreciated. The Dutch Ministry of Transport, Public Works and Water Management (RWS/RIKZ), Technology Foundation (STW), and the European Community (Environment and Climate Program) financially supported this study. J. Legler was supported by a Natural Sciences and Engineering Research Council of Canada scholarship.

Literature Cited

(1) Colborn, T.; vom Saal, F. S.; Soto, A. M. *Environ. Health Perspect.* **1993**, *101*, 378−384.
(2) Golden, R. J.; Noller, K. L.; Titus-Ernstoff, L.; Kaufman, R. H.; Mittendorf, R.; Stillman, R.; Reese, E. A. *Crit. Rev. Tox.* **1998**, *28*, 101−227.
(3) Sumpter, J. P. *Tox. Lett.* **1998**, *102−103*, 337−342.
(4) Jobling, S.; Nolan, M.; Tyler, C. R.; Brighty, G.; Sumpter, J. P. *Environ. Sci. Technol.* **1998**, *32*, 2498−2506.
(5) Lye, C. M.; Frid, C. L. J.; Gill, M. E.; McCormick, D. *Mar. Poll. Bull.* **1997**, *34*, 34−41.
(6) Allen, Y.; Scott, A. P.; Matthiessen, P.; Haworth, S.; Thain, J. E.; Feist, S. *Environ. Tox. Chem.* **1999**, *18*, 1791−1800.
(7) Tyler, C. R.; Jobling, S.; Sumpter, J. P. *Crit. Rev. Tox.* **1998**, *28*, 319−361.
(8) Zacharewski, T. R. *Environ. Sci. Technol.* **1997**, *31*, 613−623

(9) Haffter, P.; Nusslein-Volhard, C. *Int. J. Dev. Biol.* **1996**, *40*, 221−227.
(10) *Organization for Economic Development*. Guidelines for testing of chemicals; OECD Guideline 210, 1992.
(11) Legler, J.; Van den Brink, C. E.; Brouwer, A.; Murk, A. J.; Van der Saag, P. T.; Vethaak, A. D.; Van der Burg, B. *Tox. Sci.* **1999**, *48*, 55−66.
(12) Stuart, G. W.; McMurray, J. V.; Westerfield, M. *Development* **1988**, *103*, 403−412.
(13) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−254.
(14) Xia, Z.; Patino, R.; Gale, W. L.; Maule, A. G.; Densmore, L. D. *Gen. Comput. Endocrinol.* **1999**, *113*, 360−368.
(15) Todo, T.; Adachi, S.; Yamauchi, K. *Mol. Cell. Endocrinol.* **1996**, *119*, 37−45.
(16) Joore, A. T. Dissertation, University of Utrecht, The Netherlands, 1995; 188 pp.
(17) Takahashi, H. *Bull. Fac. Fish. Hokkaido Univ.* **1977**, *28*, 57−65.
(18) Desbrow, C.; Routledge, E. J.; Brighty, G. C.; Sumpter, J. P.; Waldock, M. *Environ. Sci. Technol.* **1998**, ***32***, 1549−1558.
(19) Bitman, J.; Cecil, H. C.; Harris, S. J.; Fries, G. F. *Science* **1968**, *162*, 371−372.
(20) Denison, M. S.; Chambers, J. E.; Yarbrough, J. D. *Comp. Biochem. Physiol.* **1981**, *69*, 109−112.
(21) Spearow, J. L.; Doemeny, P.; Sera, R.; Leffler, R.; Barkley, M. *Science* **1999**, *285*, 1259−1261.
(22) Tchoudakova, A.; Pathak, S.; Callard, G. V. *Gen. Comput. Endocrin.* **1999**, *113*, 388−400.
(23) Van Pelt, A. M. M.; De Rooij, D. G.; Van der Burg, B.; Van der Saag, P.; Gustafsson, J.-A.; Kuiper, G. G. J. M. *Endocrinol.* **1999**, *140*, 478−483.
(24) Saunders: P. T. K.; Fisher, J. S.; Sharpe, R. M.; Millar, M. R. *J. Endocrinol.* **1998**, *156*, 13−17.
(25) Loomis, A. K.; Thomas, P. *Biol. Reprod.* **1999**, *61*, 51−60.
(26) Sumpter, J. P.; Jobling, S. *Environ. Health Perspect.* **1995**, *103*(S7), 173−178.

Received for review March 17, 2000. Revised manuscript received July 5, 2000. Accepted July 7, 2000.

ES0000605